Biochemistry. — *Complexcoacervation in the presence of buffers and of non-electrolytes, preventing gelatination.* By H. G. BUNGENBERG DE JONG and E. G. HOSKAM. (Communicated by Prof. H. R. KRUYT.)

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1. *Introduction.*

The complex coacervation of purified gelatine and gum arabic was previously studied in detail at 40°, the pH desired of the two isohydric sols being brought about by the addition of HCl, (resp. acetic acid) [1].

So for the preparation of isohydric sols the determination of pH titration curves of the two stock sols should precede. The purpose of the following investigation is to see whether the characteristic properties of the complex coacervation (a.o. intensity of the coacervation as a function of the mixing proportion of the two isohydric sols, and the occurrence of the double valence rule on neutralization of the complex coacervation with neutral salts) are retained:

A. When buffers are used to regulate the pH,
B. When the investigation is carried out at room temperature in the presence of well-chosen non-electrolytes which prevent gelatination.

2. *Complex coacervation with buffered sols.*

In choosing a buffer we must bear in mind that the buffer salt, like any other salt, has a neutralizing effect on the complex coacervation. As the intensity of the neutralizing effect depends on the valence of the two ions — increasing with equal concentration (in m. aeq. p. L.) as the valence is higher — a buffer will preferably be selected in which there are only monovalent ions. For that reason acetate buffers were used in what follows. But since the neutralizing effect of a salt increases with the concentration of the salt, we shall have to arrange the buffers in such a way that for the variation of the pH the acetic acid concentration only is varied, that of the Na-acetate remaining constant. As the complex coacervation gelatine + gum arabic is already neutralized at 30 to 40 m. aeq. NaCl, we must choose the Na-acetate final concentration considerably lower. In what follows we choose 10 m. aeq. p. L. which is achieved by adding to 10 cc stock sol 5 cc buffer in which the Na-acetate conc. is 30 m. aeq. p. L.

The following tables give the pH of some sols buffered in this way (measurements with the H electrode at 40° C.).

The stock sols were prepared by dissolving 5 gr. airdry colloid preparation in 250 cc dist. water (further indicated as "2 % stock sol"). Next a series of buffer mixtures was prepared, consisting of 30 cc Na-acetate 0.1 N + a cc acetic acid 2 N + (70—a) dist. water.. Besides the mixtures of 10 cc stock sol + 5 cc buffer we measured mixtures of the composition of 10 cc dist. water + 5 cc buffer. The upper half of the table refers to purified colloid preparations, the lower half to unpurified preparations [2]. The results of the first series are pictured in Fig. 1.

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[1]) H. G. BUNGENBERG DE JONG and W. A. L. DEKKER, Kolloid Beihefte **43**, 143 (1935); **43**, 213 (1936).

[2]) Gelatine F00 extra of the "Lijm- en Gelatinefabriek 'Delft' " at Delft. Gum arabic: Gomme Senegal petite boule blanche I of ALLAND et ROBERT Paris: Na-nucleinicum e faece of E. MERCK. For the purification of these preparations see for isoelectric gelatine Kolloid Beihefte, **43**, 256 (1936); for Na-Arabinate and Na-Nucleinate Kolloid Beihefte **47**, 260 resp. 257 (1938).

a	pH				△ pH		
	Blank	Na Arabinate	Isoelectr. gelatine	Na Nucleinate	Na Arabinate	Isoelectr. gelatine	Na Nucleinate
2.5	4.43	4.47	4.53	4.71	+0.04	+0.10	+0.28
4	4.24*	4.28	4.35	4.50	+0.04	+0.11	+0.26
10	3.86	3.93	4.00	4.13	+0.07	+0.14	+0.27
25	3.49*	3.57	3.65*	3.76	+0.08	+0.16	+0.27
60	3.13	3.24	3.31	3.40	+0.09	+0.18	+0.27
	Blank	Gum Arabic	Gelatine	Na Nucleinicum	Gum Arabic	Gelatine	Na Nucleinicum
2.5	4.42	4.42	4.52	4.66	0	0.10	+0.24
4	4.23*	4.22	4.34	4.46	−0.01	+0.11	+0.23
10	3.86	3.87	4.00	4.11	+0.01	+0.14	+0.25
25	3.49*	3.53	3.65	3.74	+0.04	+0.16	+0.25
60	3.12	3.19	3.30	3.38	+0.07	+0.18	+0.26

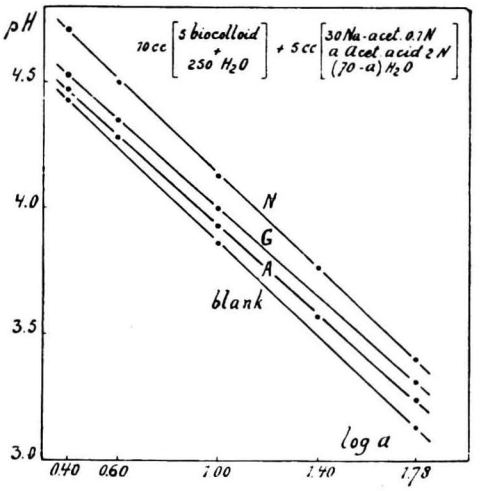

Fig. 1.

In the last three columns of the table are indicated the pH differences between the buffered sols and the buffer by itself (blank), showing that with the colloid concentration chosen here and the concentrations of the buffer components the pH of the nucleinate sols deviates most, that of the gelatine deviates less and that of the arabinate sols less again. With the relatively small final concentration of the Na-acetate these deviations are not surprising, but as has been said above it is not very well possible to increase them more, since then (at least in the gelatine-arabinate combination) the complex coacervation is too much neutralized. Better buffering can of course be obtained by choosing lower colloid concentrations.

When for instance the concentration of the stock sols is chosen 10 × smaller, the △ pH of the arabinate sols in the entire pH section will be less than 0.01 and the △ pH of the gelatine sols will also remain below 0.02. With such diluted sols it is also very well possible to demonstrate the characteristic features of the complex coacervation (e.g. with turbidity measurements), but this is not suitable for the method we have in mind

(measurement of the coacervate volume). The coacervate volume which is then separated from, for instance, 15 cc total system, becomes then very small, and for accurate measuring we should have to make use of sedimentation tubes of unpractical sizes (e.g. 150 cc).

For the purpose of investigation therefore, we have to compromise, viz. that with the simple method of experimentation the sols prepared with 10 m. aeq. acetate buffer are not exactly isohydric, but that the pH of the gelatine sol is ca. 0.1 higher than that of the arabinate sol.

Here then we can start from the two buffered sols, mixing them in different mixing proportions, or the watery sols are mixed in different mixing proportions and the buffer is added later. The first method is applied in § 4. Fig. 2 shows the results obtained by the second method (coacervate volumes noted after one night in the thermostat at 40°): a cc 2 % A (or N) + (10—a) cc 2 % G + 5 cc buffer, in which A, N and G represent the purified gelatine, Na-arabinate and Na-nucleinate preparations, the buffer consisting of 30 cc Na-acetate 0.1 N + 25 cc acetic acid 2 N added in a 100 cc measuring flask adding dist. water up to the mark.

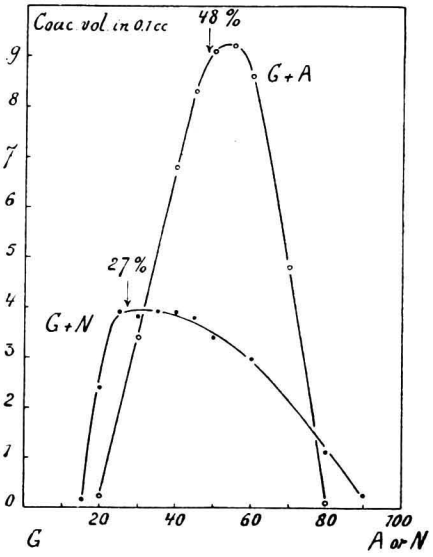

Fig. 2.

From Fig. 1 we find graphically for the pH of the corresponding unmixed sols: arabinate = 3.57, gelatine = 3.65, Na-nucleinate = 3.76. Arrows in the figure indicate the location of the electrophortically determined points of reversal of charge (48 % A in the combination G + A resp. 27 % N in the combination G + N).

As appears from Fig. 2 the points of reversal of charge do not lie exactly at, but to the left of the maximum, the coacervates on the left ascending curve branches are therefore charged positively, on the right descending branches the charge is negative.

The two curves in Fig. 2 differ in two ways, namely:

1. There is much less nucleinate (27 % N) than arabinate (48 % A) needed for the charge compensation of the gelatine.

2. The maximal coacervate volume in the G + N combination is much smaller than in the G + A combination (the waterpercentage of the G + N coacervate is much lower).

These differences are caused by the fact that the nucleinate is a colloid of much denser charge (smaller equivalent weight) than the arabinate.

In what follows we shall restrict ourselves to the complex coacervation gelatine-arabinate. The analogous combination gelatine-nucleinate is less suitable for demonstrating purposes, as here the waterpercentage of the coacervate is much lower and the coalescence of the drops to a perfectly clear layer of liquid takes place with some difficulty. Moreover there is in this combination the complication that with pH reduction of the nucleinate sol nucleine acid may form. Hence the buffered sols are clear only above ca. pH 3.7, below it they are slightly or more pronouncedly opalescent to very turbid as the pH is taken lower.

3. *Prevention of gelatination at room temperature.*

It is also possible to realize the complex coacervation at room temperature, when we see to it that there is a sufficient concentration of suitable non-electrolytes which prevent gelatination. This is a.o. possible with urea, the final concentration of which must then

be over ca. 16 %. In the presence of this it is possible to demonstrate the typical features of the complex coacervation at room temperature.

Urea however, has the following drawbacks:

1. The coacervate drops coalesce very slowly, so that a cohering coacervate layer is formed very slowly;

2. Urea has a marked "swelling" effect on the coacervate in consequence of which on the one hand the section of mixing proportions in which coacervation occurs is relatively small, on the other hand the complex coacervate is neutralized at much smaller salt concentrations than without urea. The great sensitiveness to salt therefore prevents the application here of diluted buffers to regulate the pH.

For our purposes we should dispose of a non-electrolyte which exclusively prevents gelatination and has neither a swelling, nor a condensing effect on the complex coacervate. We do not know such a substance, but we can make shift with a suitably chosen mixture of two gelatination-preventing substances, the one of which (urea) having a swelling, the other a condensing effect. The phenols generally belong to the latter category.

With a pH which is sufficiently removed from the i.e.p. of the gelatine the purpose is achieved with e.g. 10 % urea + 4 % resorcinol, in which the condensing effect of the resorcinol entirely destroys the swelling effect of the urea, so that with respect to the blank experiment (i.e. without urea + resorcinol; at 40°) the coacervates are even in a slightly condensed condition. Hence the coacervate drops rapidly coalesce and the layer is soon formed, the coacervation extends over a broad section of mixing proportions and the coacervates are evidently more resistent to neutral salts than at 40° without urea + resorcinol. Owing to the fact that the resorcinol effect becomes stronger as we approach the i.e.p. of the gelatine, resorcinol + urea are not suitable for a study of the complex coacervation in which we vary the pH systematically.

Here follow some experiments demonstrating the "opening" effect of urea and the "condensing", effect of phenols. In both cases we used stock sols of the following composition: 5 g gelatine + 6 g gum arabic + 190 cc dist. water.

In Fig. 3 A and B the composition of the mixtures for the series at 40° was a cc HCl 0.1 N + (7.5—a) cc H_2O + 5 cc stock sol. For the series at room temperature we used a stock sol prepared from the original one by adding 50 cc stock sol to 25 g urea placed in a 100 cc measuring flask, adding dist. water up to the mark. With this new stock sol mixtures were made as follows:

a cc HCl 0.1 N + (2.5—a) cc H_2O + 10 cc stock sol,

so that the final volume (12.5) cc and the colloid final concentrations were as great as in the series without urea at 40°. Fig. 3 gives the coacervate volumes after 1 night as functions of the added quantity of HCl, Fig. 3 B giving these volumes as functions of the pH measured with the H electrode.

The increase of the maximal coacervate volume and the little viscous character of the coacervate (demonstrated by tilting the sedimentation tubes) point to an "opening" effect of the urea (i.e. to a decrease of the colloid concentration in the coacervate). As additional effect there is shifting of the peak from pH 3.7 to ca. 4.0. Possibly this additional effect is to be ascribed to the HCl binding to urea which increases as the pH values become lower. Although urea is only a very weak base ($K = 1.5 \times 10^{-14}$), we can yet in our final concentration (20 % urea) reckon with the presence of some tenths of m. aeq. p. L. of urea hydrochlorid at pH 4, at pH 3 this is even some m. aeq. p. L. If in this case, as with any salts we assume a neutralizing effect, this effect increases in the direction of lower pH's and this may be one of the causes of the peak of the coacervate volume curve shifting to a higher pH.

Fig. 3 C gives the effect of some phenols on the coacervate volume at 40° C. Here we used a buffer:

(100 cc Na-acetate 0.1 N + 100 cc acetic acid 1 N + 800 cc H_2O),

the composition of the mixtures being:

a cc 0.5 mol "phenol" dissolved in buffer + (10—a) cc buffer + 5 cc stock sol (5 g + 6 A + 190 H_2O).

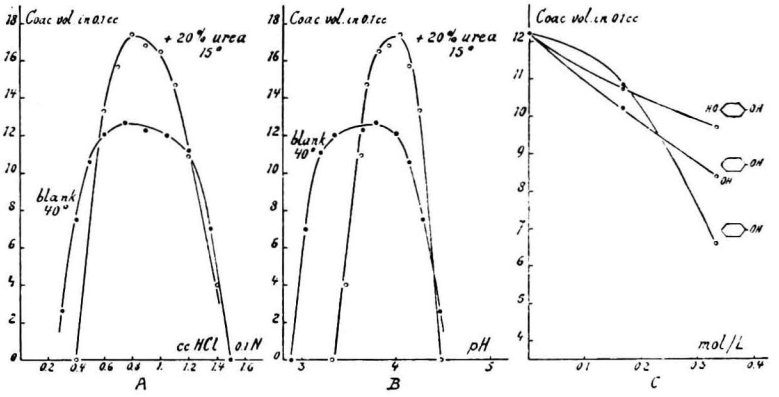

Fig. 3.

Fig. 3 C gives the results for phenol, hydrochinon and resorcinol. Also with the other phenols investigated (the effect of pyrocatechol is ca. that of resorcinol, of pyrogallol and phloroglucinol it is a little stronger, at least when the final concentration is smaller) we find: decrease of the coacervate volume and more difficult coalescibility of the coacervate drops to a clear coacervate layer. The two effects are attributable to "condensation" of the coacervate (i.e. "increase" of the colloid concentration in the coacervate). In agreement with this is also the increasing viscosity of the coacervate layer (seen when the sedimentation tubes are tilted).

4. *Simple experiments concerning complex coacervation at 40° with buffers.*

A. *Complex coacervation in an approximately isohydric mixing series.*

We first prepare a buffer by placing 200 cc Na-acetate 0.1 N + 100 cc acetic acid 2 N in a measuring flask, adding water to 1000 cc. Next we prepare separately a 4 % gelatine sol (G) and a 4 % gum arabic sol (A).

Now the two "isohydric" sols are prepared, by mixing:

1 vol. 4 % G + 1 vol. buffer
resp. 1 vol. 4 % A + 1 vol. buffer.

The two buffered sols are now filled in burettes (the one containing gelatine in a burette surrounded by a wider glass tube, filled with water of 40° C.). 4, 6, 8, 9, 10, 11, 12, 14 and 16 cc of the buffered gum arabic sol are then placed in a number of sedimentation tubes, which are then placed in a stand in the thermostat of 40° in order to heat them to the right temperature, after which 16, 14, 12, 11, 10, 9, 8, 6 and 4 cc of the buffered gelatine sol is added to them. After mixing we again place the tubes in the thermostat and after 5 minutes they are again well shaken. After some time coacervate layers begin to form in the central section and after 3 hours the coacervate volumes can already be noted. See Table and Fig. 4 A.

Electrophoretic measurements (last column of the table) show that on the left ascending branch of the curve the coacervates are positively and on the right descending branch negatively charged. The reversal of charge is here again at a mixing proportion of 50 % A, while by constructing a bisecting line the maximum of the coacervate volume curve is found to be at 51.5 % A. So here we find the same as in § 2 with purified

colloids: The reversal of charge is not exactly at but slightly to the left of the maximum of the coacervate volume curve.

cc A	cc G	% A	Coac. vol. in 0.1 cc (after 3 hrs.)	pH Measured (40°)	U (40°) (arbitrary units)
—	—	0 (100 % G)	—	3.70	—
4	16	20	0.3	—	—
6	14	30	7.0	—	—
8	12	40	13.6	3.75	+ 90
9	11	45	15.8	—	+ 56
10	10	50	17.4	3.76	+ 0
11	9	55	16.0	—	— 83
12	8	60	15.1	3.75	—122
14	6	70	9.0	—	—
16	4	80	1.2	—	—
—	—	100	—	3.82	—

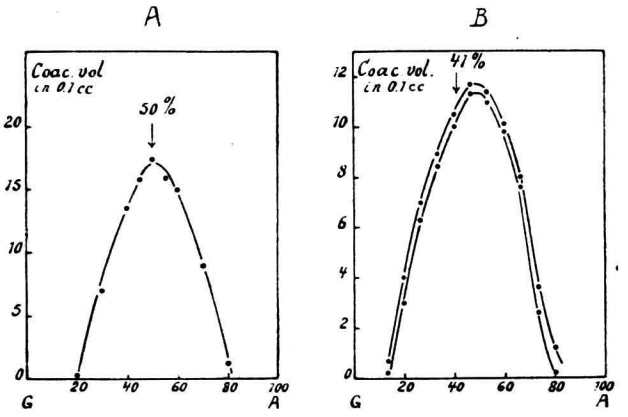

Fig. 4.

B. *Double valence rule on the neutralization of the complex coacervation with neutral salts.*

We shall here restrict ourselves to the neutralization of the coacervation with neutral salts at the optimal mixing proportion. When again we choose the final concentration of the colloids as 2 % and the Na-acetate final concentration 10 m. aeq. p. L., this is the mixing proportion of 50 % as is seen from A. In order to have sufficient space for the neutral salts added we proceed as follows: we prepare a buffer by placing 300 cc Na-acetate 0.1 N + 150 cc acetic acid 2 N in a measuring flask, distilled water is added till 1000 cc ("30 m. aeq. acetate buffer"). Then we make a mixed sol by dissolving 3 g gum arabic together with 3 g gelatine in 100 cc dist. water ("6 % A + G sol").

In sedimentation tubes we now place each time 5 cc salt solution of varying strength, then 5 cc "30 m. aeq. acetate buffer" and then 5 cc "6 % A + G sol". In these final mixtures, as in A, the Na-acetate concentration is then 10 m. aeq. p. L. and the colloid final concentration is 2 %, the mixing proportion of the two colloids being 50 %. Fig. 5 A shows the result after 1½ hours at 40°.

It is true that the sedimentation is then not yet complete, the layers growing ca. 0,03 cc

after one night, but the general character of the curve bundles does not undergo a change [1]).

So we see that the double valence rule applies to the neutralization of the complex coacervation: i.e. the salt concentrations necessary for achieving the coacervate volume = 0 (complete neutralization) are the smaller as with equal valence of the anion (monovalent) the valence of the cation increases, similar, with equal valence of the cation (monovalent) the valence of the anion increases. So the neutralizing effect decreases from left to right in following series:

$$\begin{matrix} 3-1 > 2-1 > 1-1 \\ 1-3 > 1-2 > 1-1 \end{matrix} \quad \text{"double valence rule"}$$

5. *Simple experiments concerning complex coacervation at room temperature.*

A. *Complex coacervation in an approximately isohydric mixing series.*

In each of two measuring glasses of 250 cc we put 25 g urea + 10 g resorcinol. As described in 4. we now prepare buffered gum arabic and buffered gelatine sols, adding the first to one measuring glass and the second to the other until 250 cc. We mix and leave them to cool to room temperature. The two sols are now filled in burettes and a mixing series is begun with them. Fig. 4 B gives the result after 1½ hours and after 22 hours at room temperature. The arrow indicates the location of the point of reversal of charge, determined electrophoretically. Here again we see what we noted in 3 and 4: the reversal (here at 41 % A) is near but before the maximum (here 48.5 % A) of the coacervate volume curve. As regards 3 and 4 the reversal and the maximum of the curve have both shifted to lower values of the mixing proportion. This is partly accounted for by the higher pH (compare § 2 in which we saw that in the presence of urea the pH is 0.3 higher). We could not measure any constant potential differences with the H electrode (presence of resorcinol ?) so that the glass electrode had to be used. With this we found: 100 % G = 4.07; 25 % A = 4.04; 50 % A = 4.01; 75 % A = 3.96; 100 % A = 3.92.

Hence the pH difference of the two approximately isohydric sols (= 0.15) was found to be of the order expected.

Remark.

The peak of the coacervate volume curve was 11.3 after 1½ hours, after 5 hours it was 11.6 and after 22 hours 11.7. In 4 A on the other hand we found 17.4. This difference is partly due to the different final volumes of the mixtures (in 4 A = 20 cc, here 15 cc) and to the fact that in preparing the buffered sols containing urea + resorcinol the colloid concentration had fallen below 2 %.

The decrease of the concentration we can calculate approximately from the s.g. of urea (= 1.335) and resorcinol (1.283). 25 g urea and 10 g resorcinol occupy a volume of (25 : 1.335) + (10 : 1.283) = 19 + 7.8 = 26.8 cc. This makes the colloid concentration (250—26.8) : 250 = 0.89 × the original one. When the two causes are taken into account the colloid final concentration is here 0.75 × 0.89 = 0.67 of the one in 4 A, so that the maximal coacervate volume must be 0.67 × 17.3 = 11.6, which is very near the values found. From this general agreement it is seen that with the concentration chosen of urea and resorcinol the volume diminishing effect of the resorcinol and the volume-

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[1]) The mixtures and coacervates with $K_3Fe(CN)_6$ are bluish green after one night, but this colour is not so pronounced after 1½ hours. Here we have an additional effect (oxidizing effect of $K_3Fe(CN)_6$ on the gelatine). This complication may be avoided by using $K_3CH(SO_3)_3$, the K salt of methane trisulfonic acid, which however is not so strongly neutralizing as $K_3Fe(CN)_6$, but still stronger than K_2SO_4.

increasing effect of the urea (see § 3) with the pH chosen, do indeed approximately compensate each other.

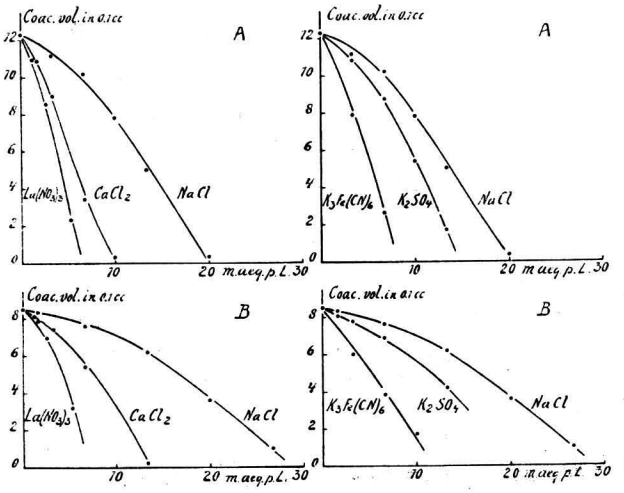

Fig. 5.

B. *Double valence rule on the neutralization of the complex coacervation with neutral salts.*

In a measuring glass of 200 cc we place 60 g urea + 24 g resorcinol, adding "6 % A + G sol" (of § 4 B) until 200 cc. Further we proceed in the same way as in 4 B, namely we again make mixtures: 5 cc salt solution + 5 cc buffer + 5 cc solmixture (containing urea + resorcinol). The results after 3 hours are pictured in Fig. 5 B. Here again we see the typical occurrence of the double valence rule:

$$3-1 > 2-1 > 1-1$$
$$1-3 > 1-2 > 1-1$$

After one night the coacervate volumes have only become a little greater, but the general character of the curve bundles remains unchanged. When we compare this experimental series with the on in 5 A we see that the neutralization by neutral salts takes place here at greater concentrations than at 40° without urea + resorcinol. At any rate the condensing resorcinol has compensated or possibly overcompensated the swelling effect of urea (which heightens the sensitiveness to salts when urea only is present). The question in how far the difference in temperature (40°—18°) affects the sensitiveness to salts remains, of course, unanswered.

Summary.

1. When studying complex coacervation it is possible to make use of diluted acetate buffers. When the effect of the pH is to be studied buffers are indicated with constant Na-acetate concentration (e.g. 10 m. aeq. p. L.) and varied acetic acid concentration.

2. Urea and resorcinol in sufficiently high concentrations neutralize the gelatination of gelatine sols. With them the complex coacervation of gelatine + gum arabic is also possible at room temperature. The strong additional effects of urea ("opening") and resorcinol (strongly condensing) however prevent their application.

3. The opening effect of 10 % urea is practically compensated by the condensing effect of 4 % resorcinol.

4. Simple experiments at 40° with buffers, and at room temperature with buffers + urea + resorcinol are indicated for the purpose of demonstrating some properties of the complex coacervation.

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